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(54) Title: INDUCTION OF IMMUNITY AGAINST TUMOR SELF–ANTIGENS

(57) Abstract

This invention provides methods and compositions for breaking tolerance to a self–antigen, especially in the context of a tumor–associated antigen. In one embodiment, the method utilized altered tumor antigens or tumor antigens derived from heterologous species to break immunological tolerance and induce a cross–reactive immune response against the corresponding native or self–antigen.

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INDUCTION OF IMMUNITY AGAINST TUMOR SELF-ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/078,889, filed March 20, 1998, the contents of which are hereby incorporated by reference.

TECHNICAL FIELD

This invention is in the field of molecular immunology and medicine. In particular, the present invention provides compositions and methods for inducing an immune response to a native self-antigen in a subject.

BACKGROUND

The goal of vaccination is to generate a protective immune response and an expanded population of memory cells ready to encounter an agent identified as foreign, which will then elicit a potent secondary immune response. T and B cells are highly antigen specific and can develop into memory cells, and therefore are the target for a successful vaccine.

Tumor specific T cells, derived from cancer patients, will bind and lyse tumor cells. This specificity is based on their ability to recognize short amino acid sequences (epitopes) presented on the surface of the tumor cells by MHC class I and class II molecules. These epitopes are derived from the proteolytic degradation of intracellular proteins called tumor antigens encoded by genes that are either uniquely or aberrantly expressed in tumor or cancer cells.

The availability of specific anti-tumor T cells has enabled the identification of tumor antigens and subsequently the generation of cancer vaccines designed to provoke an anti-tumor immune response. A critical target of vaccines is the specialized antigen-presenting cell ("APC"), the most

immunologically powerful of which is the bone marrow-derived dendritic cell ("DC").

DCs are potent antigen presenters that express high levels of co-stimulatory molecules and are capable of activating both $CD4^+$ and $CD8^-$ naive T lymphocytes. Results obtained in several animal models have shown that DCs pulsed with defined tumor-associated peptides or with peptides eluted from the surface of tumor cells are capable of inducing an antigen-specific CTL response resulting in protection from tumor challenge and, in some instances, regression of established tumors. The same type of approach has also been tested in human clinical trials with encouraging results. For example, Hsu et al. have reported that four B cell lymphoma patients infused with autologous DCs pulsed with tumor-specific idiotype protein all developed an idiotype-specific proliferative response accompanied by complete tumor regression in two patients and partial regression in a third. Hsu et al. (1996) Nature Med. **2**:52. More recently, Nestle et al. reported that melanoma patients treated with autologous DCs pulsed with tumor lysate or a cocktail of CTL peptide epitopes, developed cell-mediated immunity with objective clinical responses in 5 out of 16 patients evaluated. Nestle et al. (1998) Nature Med. **4**:328.

Successful cancer therapy, similar to the ones noted above, is rare. The high incidence of failure may be due to the fact that naturally occurring neoplasms do not possess antigens that can serve as inducers and/or targets for a tumor destructive immune response, although immunological reactions mediated by either lymphocytes or antibodies to cultivated human tumors have been reported. Hellstrom K. and Hellstrom I. (1969) Adv. Cancer Res. **12**:167. Indeed, mechanisms of systemic immune tolerance to self have begun to emerge, particularly from studies in transgenic mouse systems. Hanahan D. (1990) Ann. Rev. Cell Biol. **6**:493. Mechanisms of systemic immune tolerance include deletion of potentially autoreactive B or T cells, induction of anergy in B and T cells, and the poorly defined phenomenon of suppression of immune response by suppressor cells. Houghton and Lewis, pages 37-54 in Forni, et al., eds. (1994) CYTOKINE-INDUCED TUMOR IMMUNOGENICITY, Academic Press, New York.

Thus, a need exists to overcome immune tolerance to self-antigens and to provide an effective cancer vaccine. This invention satisfies these needs and provides related advantages as well.

DISCLOSURE OF THE INVENTION

In the present invention, immunization is carried out with a heterologous antigen or an altered antigen that is structurally distinct from the self-antigen or "native" antigen yet is still capable of inducing an immune response against the self-antigen. Such antigens are immunogenic (seen as foreign) and serve to induce an immune response that cross-reacts with the native antigen.

In the context of cancer gene therapy, the invention comprises using modified (altered) tumor antigens or tumor antigens derived from heterologous species to break immunological tolerance and induce a cross-reactive immune response against the corresponding native or self-antigen. For example, immunizing humans against the human melanoma antigen gp100 requires breaking tolerance against a self-antigen. As shown below, the use of the non self-antigen can provide protective immunity and tumor reduction *in vivo*. Immunization and therapy are accomplished by any of the following methods: 1) administration of a vector encoding altered tumor antigen or antigen from a heterologous species; 2) infecting dendritic cells *ex vivo* or *in vivo* with the same vector; or 3) use of transduced dendritic cells or APCs to stimulate production of an enriched population of antigen-specific immune effector cells that can be adoptively transferred into the host.

Antigen presenting cells such as dendritic cells also are useful to expand a population of immune effector cells that specifically recognize and lyse the cells presenting the heterologous antigen and its native or self-counterpart. The expanded immune effector cell populations and their use in prophylactical and therapeutical methods also are provided herein.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A through 1D show the results of immunizing mice with syngeneic DCs. Five female C57BL/6 mice (represented by five different symbols in the panels) were immunized with B16 melanoma using dendritic cells transfected with Ad vector encoding homologous mouse gp100 versus heterologous human gp100.

Figures 2A through 2C show induction of CTL activity following immunization with Ad2/hugp100v1 vector or Ad2/hugp100v1-transduced DCs. Spleens from groups of 3 animals were collected 15 days after i.v. administration of vehicle (Figure 2A), Ad2/hugp100v1-transduced DCs (Figure 2B) or i.d. delivery of Ad2/hugp100v1 vector (Figure 2C). Pooled spleen cells from each group were re-stimulated *in vitro* with syngeneic SVB6KHA fibroblasts transduced with Ad2/hugp100v 1 and were tested for cytolytic activity after 6 days of culture. Targets consisted of B16 cells and SVB6KHA fibroblasts untransduced or transduced with Ad2/hugp100v 1 or wild type Ad2 deleted for E3 (SVB6KHA-Ad2Δ2.9). Figure legend: (-●- B16; -■- SVB6KHA - Untransduced; -▲- SVB6KHA-Ad2/hugp100v1; -◆- SVB6HA-Ad2Δ2.9).

Figure 3 compares the effectiveness of immunization with DCs transduced with Ad vector encoding various melanoma-associated antigens. The figure shows the evaluation of the nature of the antigen. Figure legend: (-□- Untransduced DCs; -◇- Ad2/hugp100v1 DCs; -○- Ad2mgp100 DCs; -Δ- Ad2/mTRP-2 DCs). Groups of 5 C57BL/6 mice were injected i.v. with $5x10^5$ DCs that were either untransduced or transduced with Ad2/hugp100v1, Ad2/mgp100 or Ad2/mTRP-2 vector. The animals were challenged 15 days later with a s.c. injection of $2x10^4$ B16 melanoma cells.

Figure 4 shows the frequency of gp100-reactive splenic T lymphocytes following immunization with Ad2/hugp100- or Ad2/empty vector-transduced DCs. Spleen cells from immunized mice were stimulated in vitro with a cytotoxic T lymphocyte peptide epitope derived from hugp100 (open bar); or the corresponding epitope from mgp100 (solid bar). An ovalbumin-derived epitope

was used as a negative control (hatched bar). The number of T lymphocytes that produced γ-interferon upon recognition of peptide was measured by elispot.

MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Definitions

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology, microbiology, cell biology and recombinant DNA, which are within the skill of the art. See, *e.g.*, Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1989) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

As used herein, certain terms may have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "genetically modified" means containing and/or expressing a foreign gene or nucleic acid sequence which in turn, modifies the genotype or phenotype of the cell or its progeny. In other words, it refers to any addition, deletion or disruption to a cell's endogenous nucleotides.

As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or

proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1α), interleukin-11 (IL-11), MIP-1α, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

The term "antigen presenting cell" ("APC"), as used herein, intends any cell which presents on its surface an antigen in association with a major histocompatibility complex molecule, or portion thereof, or, alternatively, one or more non-classical MHC molecules, or a portion thereof. Examples of suitable APCs are discussed in detail below and include, but are not limited to, whole cells such as macrophages, dendritic cells, B cells, hybrid APCs, and foster antigen presenting cells or other cell type(s) expressing the necessary MHC and co-stimulatory molecules. Methods of making hybrid APCs have been described. See, for example, International Patent Application Publication Nos. WO 98/46785 and WO 95/16775.

Dendritic cells (DCs) are potent antigen-presenting cells. It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell

activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called co-stimulatory signals, is neither antigen-specific nor MHC-restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals. As used herein, "dendritic cell" is to include, but not be limited to a pulsed dendritic cell, a foster cell , a dendritic cell hybrid or a genetically modified dendritic cell. Methods for generating dendritic cells from peripheral blood or bone marrow progenitors have been described (Inaba et al. (1992) J. Exp. Med. **175**:1157; Inaba et al. (1992) J. Exp. Med. **176**:1693-1702; Romani et al. (1994) J. Exp. Med. **180**:83-93; Sallusto et al. (1994) J. Exp. Med. **179**:1109-1118; Bender et al. (1996) *J. Imm. Methods* **196**:121-135; and Romani et al. (1996) *J. Imm. Methods* **196**:137-151).

"Co-stimulatory molecules" are molecules involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. "Co-stimulatory activity" was originally defined as an activity provided by bone-marrow-derived accessory cells such as macrophages and dendritic cells, the so called "professional" APCs. Several molecules have been shown to enhance co-stimulatory activity. These are heat stable antigen (HSA) (Liu Y. et al. (1992) J. Exp. Med. **175**:437), chondroitin sulfate-modified MHC invariant chain (Ii-CS) (Naujokas M.F. et al. (1993) Cell **74**:257), intracellular adhesion molecule 1 (ICAM-1) (Van Seventer G.A. (1990) J. Immunol. **144**:4579), B7-1 and B7-2/B70 (Schwartz R.H. (1992) Cell **71**:1065) and B7's counter-receptor CD28 or CTLA-4 on T cells (Freeman et al. (1993) Science **262**:909; Young et al. (1992) J. Clin. Invest. **90**: 229; and Nabavi et al. (1992) Nature **360**:266). Other important co-stimulatory molecules are CD40, CD54, CD80, CD86. As used herein, the term "co-stimulatory molecule" encompasses any single molecule or combination of molecules which, when acting together with a peptide/MHC complex bound by a TCR on the surface of a T cell, provides a co-stimulatory effect which achieves activation of the T cell that binds the peptide. The term thus encompasses B7, or other co-stimulatory molecule(s) on an antigen-presenting matrix such as an APC, fragments thereof (alone,

complexed with another molecule(s), or as part of a fusion protein) which, together with peptide/MHC complex, binds to a cognate ligand and results in activation of the T cell when the TCR on the surface of the T cell specifically binds the peptide. Co-stimulatory molecules are commercially available from a variety of sources, including, for example, Beckman Coulter. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified co-stimulatory molecules (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

The term "antigen" is used in its broadest sense and includes minimal epitopes and chimeric molecules in addition to isolated full length proteins. A "self-antigen", also referred to herein as a "native antigen", is an antigenic peptide that induces little or no immune response in the subject due to self-tolerance to the antigen. An example of a self-antigen is the melanoma antigen gp100. The antigen of this vaccine is "heterologous" (i.e., allogeneic or a homologe from an isolated species, e.g., a murine antigen administered to a human patient) or an "altered antigen" as compared to the corresponding native self-antigen. The heterologous or altered antigen also can be made by chemical synthesis.

The term "immune effector cells" refers to cells capable of binding an antigen or which mediate an immune response. These cells include, but are not limited to, T cells, B cells, monocytes, macrophages, NK cells and cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates. Certain diseased tissues express specific antigens and CTLs specific for these antigens have been identified. For example, approximately 80% of melanomas express the antigen known as gp100.

The term "immune effector molecule" as used herein, refers to molecules capable of antigen-specific binding, and includes antibodies, T cell antigen receptors, and MHC Class I and Class II molecules.

A "naïve" immune effector cell is an immune effector cell that has never been exposed to an antigen.

As used herein, the term "educated, antigen-specific immune effector cell" is an immune effector cell as defined above, which has encountered antigen and which is specific for that antigen. An educated, antigen-specific immune effector cell may be activated upon binding antigen. "Activated" implies that the cell is no longer in G_0 phase, and begins to produce cytokines characteristic of the cell type. For example, activated CD4+ T cells secrete IL-2 and have a higher number of high affinity IL-2 receptors on their cell surfaces relative to resting CD4+ T cells.

A peptide or polypeptide of the invention may be preferentially recognized by antigen-specific immune effector cells, such as B cells and T cells. In the context of T cells, the term "recognized" intends that a peptide or polypeptide of the invention, comprising one or more synthetic antigenic epitopes, is recognized, i.e., is presented on the surface of an APC together with (i.e., bound to) an MHC molecule in such a way that a T cell antigen receptor (TCR) on the surface of an antigen-specific T cell binds to the epitope wherein such binding results in activation of the T cell. The term "preferentially recognized" intends that a polypeptide of the invention is substantially recognized, as defined above, by a T cell specific for an antigen. Assays for determining whether an epitope is recognized by an antigen-specific T cell are known in the art and are described herein.

The term "syngeneic" or "autologous" as used herein, indicates the origin of a cell. Thus, a cell being administered to an individual (the "recipient") is autogeneic if the cell was derived from that individual (the "donor") or a genetically identical individual. An syngeneic cell can also be a progeny of an syngeneic cell. The term also indicates that cells of different cell types are derived from the same donor or genetically identical donors. Thus, an effector cell and an antigen presenting cell are said to be syngeneic if they were derived from the same donor or from an individual genetically identical to the donor, or if they are progeny of cells derived from the same donor or from an individual genetically identical to the donor.

Similarly, the term "allogeneic" as used herein, indicates the origin of a cell. Thus, a cell being administered to individual (the "recipient") is allogeneic if

the cell was derived from an individual not genetically identical to the recipient; in particular, the term relates to non-identity in expressed MHC molecules. An allogeneic cell can also be a progeny of an allogeneic cell. The term also indicates that cells of different cell types are derived from genetically non-identical donors, or if they are progeny of cells derived from genetically non-identical donors. For example, an APC is said to be allogeneic to an effector cell if they are derived from genetically non-identical donors.

A "hybrid" cell refers to a cell having both antigen presenting capability and also expresses one or more specific antigens. In one embodiment, these hybrid cells are formed by fusing, *in vitro*, APCs with cells that are known to express the one or more antigens of interest.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (either morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989) *Supra*). Similarly, an eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be

obtained commercially or assembled by the sequences described in methods well known in the art, for example, the methods described below for constructing vectors in general.

The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an α chain encoded in the MHC associated noncovalently with β2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to $CD8^+$ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated α and β chains. Class II MHC are known to participate in antigen presentation to $CD4^+$ T cells and, in humans, include HLA-DP, -DQ, and DR. The term "MHC restriction" refers to a characteristic of T cells that permits them to recognize antigen only after it is processed and the resulting antigenic peptides are displayed in association with either a self class I or class II MHC molecule. Methods of identifying and comparing MHC are well known in the art and are described in Allen M. et al. (1994) Human Imm. **40**:25; Santamaria P. et al. (1993) Human Imm. **37**:39 and Hurley C.K. et al. (1997) Tissue Antigens **50**:401.

The term "peptide" is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, *e.g.* ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an

oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a solid support, a detectable agent or label) or active, such as an adjuvant.

As used herein, "solid phase support" or "solid support" used interchangeably, is not limited to a specific type of support. Rather a large number of supports are available and are known to one of ordinary skill in the art. Solid phase supports include silica gels, resins, derivatized plastic films, glass beads, cotton, plastic beads, and alumina gels. As used herein, "solid support" also includes synthetic antigen-presenting matrices, cells, and liposomes. A suitable solid phase support may be selected on the basis of desired end use and suitability for various protocols. For example, for peptide synthesis, solid phase support may refer to resins such as polystyrene (*e.g.*, PAM-resin obtained from Bachem Inc., Peninsula Laboratories, etc.), POLYHIPE® resin (obtained from Aminotech, Canada), polyamide resin (obtained from Peninsula Laboratories), polystyrene resin grafted with polyethylene glycol (TentaGel®, Rapp Polymere, Tubingen, Germany) or polydimethylacrylamide resin (obtained from Milligen/Biosearch, California).

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages.

The term "immunomodulatory agent" as used herein, is a molecule, a macromolecular complex, or a cell that modulates an immune response and encompasses a synthetic antigenic peptide of the invention alone or in any of a variety of formulations described herein; a polypeptide comprising a synthetic antigenic peptide of the invention; a polynucleotide encoding a peptide or polypeptide of the invention; a synthetic antigenic peptide of the invention bound to a Class I or a Class II MHC molecule on an antigen-presenting matrix, including an APC and a synthetic antigen-presenting matrix (in the presence or absence of co-stimulatory molecule(s)); a synthetic antigenic peptide of the invention covalently or non-covalently complexed to another molecule(s) or macromolecular structure; and an educated, antigen-specific immune effector cell which is specific for a peptide of the invention.

The term "modulate an immune response" includes inducing (increasing, eliciting) an immune response; and reducing (suppressing) an immune response. An immunomodulatory method (or protocol) is one that modulates an immune response in a subject.

As used herein, the term "inducing an immune response in a subject" is a term well understood in the art and intends that an increase of at least about 2-fold, more preferably at least about 5-fold, more preferably at least about 10-fold, more preferably at least about 100-fold, even more preferably at least about 500-fold, even more preferably at least about 1000-fold or more in an immune response to an antigen (or epitope) can be detected (measured), after introducing the antigen (or epitope) into the subject, relative to the immune response (if any) before introduction of the antigen (or epitope) into the subject. An immune response to an antigen (or epitope), includes, but is not limited to, production of an antigen-specific (or epitope-specific) antibody, and production of an immune cell expressing on its surface a molecule which specifically binds to an antigen (or epitope). Methods of determining whether an immune response to a given antigen (or epitope) has been induced are well known in the art. For example, antigen-specific antibody can be detected using any of a variety of immunoassays known in the art, including, but not limited to, ELISA, wherein, for example, binding of

an antibody in a sample to an immobilized antigen (or epitope) is detected with a detectably-labeled second antibody (e.g., enzyme-labeled mouse anti-human Ig antibody). Immune effector cells specific for the antigen can be detected any of a variety of assays known to those skilled in the art, including, but not limited to, ^{51}Cr-release assays, ^{3}H-thymidine uptake assays or induction of cytokine release.

As used herein, the term "a disease or condition related to a population of $CD4^+$ or $CD8^+$ T cells" is one which can be related to a population of $CD4^+$ or CD8+ T cells, such that these cells are primarily responsible for the pathogenesis of the disease; it is also one in which the presence of $CD4^+$ or $CD8^+$ T cells is an indicia of a disease state; it is also one in which the presence of a population $CD4^+$ or $CD8^+$ T cells is not the primary cause of the disease, but which plays a key role in the pathogenesis of the disease; it is also one in which a population of $CD4^+$ or $CD8^+$ T cells mediates an undesired rejection of a foreign antigen. Examples of a condition related to a population of $CD4^+$ or $CD8^+$ T cells include, but are not limited to, autoimmune disorders, graft rejection, immunoregulatory disorders, and anaphylactic disorders.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without treatment or prevention as described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

"Host cell" or "recipient cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules, polynucleotides and/or peptides (or

polypeptides). It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be procaryotic or eucaryotic, and include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

The term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. For example, with respect to a polynucleotide, an isolated polynucleotide is one that is separated from the 5' and 3' sequences with which it is normally associated in the chromosome. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated" "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally occurring counterpart by its primary sequence, or alternatively, by another characteristic such as glycosylation pattern. Although not explicitly stated for each of the inventions disclosed herein, it is to be understood that all of the above embodiments for each of the compositions disclosed below and under the appropriate conditions, are provided by this invention. Thus, a non-naturally occurring polynucleotide is provided as a separate embodiment from the isolated naturally occurring polynucleotide. A protein produced in a bacterial cell is provided as a separate embodiment from the naturally occurring protein isolated from a eukaryotic cell in which it is produced in nature.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

This invention provides improved cancer vaccines and methods of using the vaccines to induce an immune response to a native self-antigen in a subject. As shown in the experimental examples below, the compositions and methods of this invention provide protective immunity against growth of tumor cells *in vivo* and a means to inhibit the growth of tumors *in vivo*. The methods also induce tumor reduction of established tumors *in vivo*. For purposes of immunization, heterologous/altered antigens can be delivered to antigen-presenting cells as protein/peptide or in the form of polynucleotide encoding the protein/peptide. Antigen-presenting cells (APCs), as defined above, include but are not limited to dendritic cells (DCS), monocytes/macrophages, B-lymphocytes or other cell type(s) expressing the necessary MHC/co-stimulatory molecules. The methods described below focus primarily on DCS which are the most potent, preferred APCs.

This invention also provides an isolated novel heterologous or altered antigen that is capable of inducing an immune response against a self-antigen in a subject, an isolated nucleic acid encoding the antigen, as well as vectors and host cells containing the nucleic acids. Methods of replicating and expressing the isolated nucleic acids also are within the scope of this invention. Vectors and methods for *in vitro* and *in vivo* transduction are briefly described below and are well known in the art. The incorporation and expression of the exogenous nucleic

acid can be confirmed using RT-PCR, Northern and Southern blotting analysis. Sambrook et al. (1989) *Supra.*

The methods of the invention are exemplified below. Melanoma-associated antigens (MAAs) were used to transduce murine DCs which were then tested for their ability to activate cytotoxic T lymphocytes (CTLs) and induce protective immunity against B16 melanoma tumor cells. Dendritic cells derived from bone marrow displayed surface markers characteristic of DCs and were functionally active *in vitro* as determined in a mixed lymphocyte reaction and as indicated by their ability to induce primary antigen-specific proliferation of syngeneic T lymphocytes. The DCs were efficiently transduced with adenovirus type 2 (Ad2) based vectors while remaining phenotypically and functionally intact. Immunization of C57BL/6 mice with DCs transduced with Ad vector encoding the non-self human gp100 melanoma antigen (Ad2/hugp100) elicited the development of gp100-specific CTLs capable of lysing syngeneic fibroblasts transduced with Ad2/hugp100 as well as B16 cells expressing endogenous murine gp100. The induction of gp100-specific CTLs was associated with long-term protection against lethal subcutaneous challenge with B16 cells.

Although this invention is exemplified using heterologous gp100 melanoma tumor antigen, any heterologous or altered antigen is useful in the methods described herein.

For example, polypeptides and the polynucleotides encoding antigens of this invention can be, in one embodiment, the heterologous counterpart or an altered antigen of previously characterized tumor-associated antigens such as MUC-1 (Henderson et al. (1996) Cancer Res. **56**:3763); MART-1 (Kawakami et al. (1994) Proc. Natl. Acad. Sci. **91**:3515; Kawakami et al. (1997) Intern. Rev. Immunol. **14**:173; Ribas et al. (1997) Cancer Res. **57**:2865); HER-2/neu (U.S. Patent No. 5,550,214); MAGE (PCT/US92/04354); HPV16, 18E6 and E7 (Ressing et al. (1996) Cancer Res. **56(1)**:582; Restifo (1996) Current Opinion in Immunol. **8**:658; Stern (1996) Adv. Cancer Res. **69**:175; Tindle et al. (1995) Clin. Exp. Immunol. **101**:265; van Driel et al. (1996) Annals of Medicine **28**:471); CEA (U.S. Patent No. 5,274,087); PSA (Lundwall, A. (1989) Biochem. Biophys.

Research Communications **161**:1151); prostate membrane specific antigen (PSMA) (Israeli et al. (1993) Cancer Research **53**:227); tyrosinase (U.S. Patent Nos. 5,530,096 and 4,898,814; Brichard et al. (1993) J. Exp. Med. **178**:489); tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2); NY-ESO-1 (Chen et al. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**:1914), or the GA733 antigen (U.S. Patent No. 5,185,254).

Also within the scope of this invention is an heterologous or altered antigen corresponding to an epitope or wild-type antigenic peptide corresponding to a yet unidentified protein. A common strategy in the search for tumor antigens is to isolate tumor-specific T-cells and attempt to identify the antigens recognized by these cells. In patients with cancer, specific CTLs have been derived from lymphocytic infiltrates present at the tumor site. Weidmann et al., *supra*. These TILs are unique cell population that can be traced back to sites of disease when they are labeled with indium and adoptively transferred. Alternatively, large libraries of putative antigens can be produced and tested. Using the "phage method" (Scott and Smith (1990) Science **249**:386; Cwirla et al. (1990) Proc. Natl. Acad. Sci. **87**:6387; and Devlin et al. (1990) Science **249**:404), very large libraries can be constructed. Another approach uses primarily chemical methods, of which the Geysen method (Geysen et al. (1986) Mol. Immunol. **23**:709; and Geysen et al. (1987) J. Immunol. Method **102**:259) and the method of Fodor et al. (1991) Science **251**:767, are examples. Furka et al. (1988) 14th Inter. Cong. Bio. Vol. **5**, Abst. FR:013; Furka (1991) Inter. J. Peptide Protein Res. **37**:487), Houghton (U.S. Patent No. 4,683,211, issued December 1986) and Rutter, et al. (U.S. Patent No. 5,010,175, issued April 23, 1991) describe methods to produce a mixture of peptides.

In a further aspect of this invention, Solid-**PH**ase Epitope **RE**covery ("SPHERE", described in PCT WO 97/35035) described below, can be used to identify tumor antigens and altered antigens corresponding to self antigens.

After identification and cloning of an altered antigen, the antigen or epitope can be expressed and purified for presentation to APC using the methods disclosed herein. In a further embodiment, the full-length native antigen can be

selectively modified to encode or present the altered epitope using methods known in the art, e.g. PCR directed mutagenesis. Sambrook et al., *supra*.

This invention further provides methods to elicit $CD4^+$ and $CD8^+$ T cells responses in a subject. The induction of this immune response also is a means to assay a positive response to the therapy. The presence of a large number of T-cells in tumor has been correlated with a prognostically favorable outcome in some cases (Whiteside and Parmiani (1994) Cancer Immunol. Immunother. **39**:15). Woolley et al. (1995) Immmunology **84**:55, has shown that implantation of polyurethane sponges containing irradiated tumor cells can efficiently trap anti-tumor CTLs (4-times greater than lymph fluid, 50-times greater than spleen or peripheral blood). Following activation with T-cell cytokines in the presence of their appropriately presented recognition antigen, TILs proliferate in culture and acquire potent anti-tumor cytolytic properties. Weidmann et al. (1994) Cancer Immunol. Immunother. **39**:1. Assays to determine T cell response are well known in the art and any method that will compare T cell number and activity prior to and subsequent to therapy can be utilized. In addition, the induction of co-stimulatory cytokines by the heterologous/altered antigen could also stimulate pre-existing anergic or low affinity self-reactive CTL clones.

When the method is practiced *in vitro* as a screen to identify antigenic peptides and nucleic acids of the invention, induction of cytotoxic T lymphocytes capable of lysing host tumor cells indicates that the antigenic peptide and/or nucleic acid of the screen is a potential therapeutic agent.

The methods of this invention can be further modified by co-administering more than one heterologous/altered antigen and/or an effective amount of a cytokine or co-stimulatory molecule or other transgene to the subject.

The antigen is administered to the subject either as a nucleic acid coding for the peptide/protein or by administering APC presenting the antigen. In one embodiment, the APC is a dendritic cell which includes, but is not limited to a pulsed or genetically modified dendritic cell. When the method is practiced *in vitro*, the APC may be a foster antigen presenting cell. Methods of presenting the antigen to the APC are described herein.

The APC can be further genetically modified to co-express a cytokine alone, or in combination with a co-stimulatory molecule or other transgene.

The APC expressing a heterologous and/or altered antigen also can be used to expand and isolate a population of immune effectors which, in turn, are useful for adoptive immunotherapy alone or as an adjuvant to the methods described above. As above, cytokines and/or co-stimulatory molecules or nucleic acids encoding them, can be co-administered with the immune effector cells. Alternatively, the immune effector cells can be genetically modified to express a foreign nucleic acid encoding a cytokine or co-stimulatory molecule. Prior to administration *in vivo*, the immune effector cells are screened *in vitro* for their ability to lyse tumor cells.

Furthermore, the invention provides a method for cloning the cDNA and genomic DNA encoding such protein by generating degenerate oligonucleotides probes or primers based on the sequence of the epitope. Compositions comprising the nucleic acid and a carrier, such as a pharmaceutically acceptable carrier, a solid support or a detectable label, are further provided by this method as well as methods for detecting the sequences in a sample using methods such as Northern analysis, Southern analysis and PCR.

Further provided by this invention are therapeutic and diagnostic comprising oligopeptide sequences determined according to the foregoing methods. Compositions comprising the oligopeptide sequence and a carrier, such as a pharmaceutically acceptable carrier, a solid support or a detectable label, are further provided by this method as well as methods for detecting the oligopeptide sequence in a sample using methods such as Western analysis and ELISA. Harlow and Lane (1989) *supra*.

Materials and Methods

Identification of Tumor Associated Antigens

Any conventional method, e.g., subtractive library, comparative Northern and/or Western blot analysis of normal and tumor cells, Expression Cloning, Serial Analysis Gene Expression "SAGE" (U.S. Patent No. 5,695,937) and Solid

PHase Epitope REcovery "SPHERE" (described in PCT WO 97/35035), can be used to identify putative antigens for use in the subject invention.

SAGE analysis can be employed to identify the antigens recognized by expanded immune effector cells such as CTLs. SAGE analysis involves identifying nucleotide sequences expressed in the antigen-expressing cells. Briefly, SAGE analysis begins with providing complementary deoxyribonucleic acid (cDNA) from (1) the antigen-expressing population and (2) cells not expressing that antigen. Both cDNAs can be linked to primer sites. Sequence tags are then created, for example, using the appropriate primers to amplify the DNA. By measuring the differences in these tags between the two cell types, sequences which are over expressed in the antigen-expressing cell population can be identified.

Expression cloning methodology as described in Kawakami et al. (1994) PNAS **91**:3515, also can be used to identify a novel tumor-associated antigen. Briefly, in this method, a library of cDNAs corresponding to mRNAs derived from tumor cells is cloned into an expression vector and introduced into target cells which are subsequently incubated with cytotoxic T cells. One identifies pools of cDNAs that are able to stimulate the CTL and through a process of sequential dilution and re-testing of less complex pools of cDNAs one is able to derive unique cDNA sequences that are able to stimulate the CTL and thus encode the cognate tumor antigen.

An antigen identification method, **SPHERE**, is described in PCT WO 97/35035. Briefly, an empirical screening method for the identification of MHC Class I-restricted CTL epitopes is described that utilizes peptide libraries synthesized on a solid support (*e.g.*, plastic beads) where each bead contains approximately 200 picomoles of a unique peptide that can be released in a controlled manner. The synthetic peptide library is tailored to a particular HLA restriction by fixing anchor residues that confer high-affinity binding to a particular HLA allele (*e.g.*, HLA-A2) but contain a variable TCR epitope repertoire by randomizing the remaining positions. Roughly speaking, 50 96-well plates with 10,000 beads per well will accommodate a library with a complexity

of approximately 5 X 10^7. In order to minimize both the number of CTL cells required per screen and the amount of manual manipulations, the eluted peptides can be further pooled to yield wells with any desired complexity. Based on experiments with soluble libraries, it should be possible to screen 10^7 peptides in 96-well plates (10,000 peptides per well) with as few as 2 X 10^6 CTL cells. After cleaving a percentage of the peptides from the beads and incubating them with ^{51}Cr-labeled APCs (*e.g.*, T2 cells) and the CTL line(s), peptide pools containing reactive species can be determined by measuring ^{51}Cr-release according to standard methods known in the art. Alternatively, cytokine production (*e.g.*, interferon-γ) or proliferation (e.g., incorporation of ^{3}H-thymidine) assays may be used. After identifying reactive 10,000-peptide mixtures, the beads corresponding to those mixtures are separated into smaller pools and distributed to new 96-well plates (*e.g.*, 100 beads per well). An additional percentage of peptide is released from each pool and re-assayed for activity by one of the methods listed above. Upon identification of reactive 100-peptide pools, the beads corresponding to those peptide mixtures are redistributed at 1 bead per well of a new 96-well plate. Once again, an additional percentage of peptide is released and assayed for reactivity in order to isolate the single beads containing the reactive library peptides. The sequence of the peptides on individual beads can be determined by sequencing residual peptide bound to the beads by, for example, N-terminal Edman degradation or other analytical techniques known to those of skill in the art.

In vitro confirmation of the immunogenicity of a putative antigen of this invention can be confirmed using the method described below which assays for the induction of CTLs.

After isolation of the epitope or antigen, it can be expressed and purified using methods known in the art.

Alternatively, muteins of the antigen as well as allogeneic and antigens from a different species, of previously characterized antigens are useful in the subject invention. For example, MART1 and gp100 are melanocyte differentiation antigens specifically recognized by HLA-A2 restricted tumor-

infiltrating lymphocytes (TILs) derived from patients with melanoma, and appear to be involved in tumor regression (Kawakami Y. et al. (1994) PNAS USA **91**:6458 and Kawakami Y. et al. (1994) PNAS USA **91**:91:3515). Recently, the mouse homologue of human MART-1 has been isolated. The full-length open reading frame of the mouse MART1 consists of 342 bp, encoding a protein of 113 amino acid residues with a predicted molecular weight of ~13 kDa. Alignment of human and murine MART1 amino acid sequences showed 68.6% identity.

The murine homologe of gp100 has also been identified. The open reading frame consists of 1,878 bp, predicting a protein of 626 amino acid residues which exhibits 75.5% identity to human gp100.

Additional antigens include, but are not limited to HER-2/neu (U.S. Patent No. 5,550,214); MAGE (PCT/US92/04354); HPV16, 18E6 and E7 (Ressing et al. (1996) Cancer Res. **56(1)**:582; Restifo (1996) Current Opinion in Immunol. **8**:658; Stern (1996) Adv. Cancer Res. **69**:175; Tindle et al. (1995) Clin. Exp. Immunol. **101**:265; and van Driel et al. (1996) Annals of Medicine **28**:471); CEA (U.S. Patent No. 5,274,087); PSA (Lundwall A. (1989) Biochem. Biophys. Research Communications **161**:1151); prostate membrane specific antigen (PSMA) (Israeli et al. (1993) Cancer Research **53**:227); tyrosinase (U.S. Patent Nos. 5,530,096 and 4,898,814, and Brichard et al. (1993) J. Exp. Med. **178**:489); tyrosinase related proteins 1 or 2 (TRP-1 and TRP-2); NY-ESO-1 (Chen et al. (1997) PNAS **94**:1914); or the GA733 antigen (U.S. Patent No. 5,185,254).

In vitro confirmation of the immunogenicity of a putative antigen of this invention can be confirmed using the method described below which assays for the generation of CTLs.

Isolation, Culturing and Expansion of APCs, Including Dendritic Cells

Various methods to isolate and characterize APCs including DCs have been known in the art. At least two methods have been used for the generation of human dendritic cells from hematopoietic precusor cells in peripheral blood or bone marrow. One approach utilizes the rare CD34+ precursor cells and stimulate them with GM-CSF plus TNF-α. The other method makes use of the more

abundant CD34- precursor population, such as adherent peripheral blood monocytes, and stimulate them with GM-CSF plus IL-4 (see, for example, Sallusto et al. (1994), *supra*).

In one aspect of the invention, the method described in Romani et al (1996), *supra*; and Bender et al (1996), *supra* is used to generate both immature and mature dendritic cells from the peripheral blood mononuclear cells (PBMC) of a mammal, such as a murine, simian or human. Briefly, isolated PBMC are pre-treated to deplete T- and B-cells by means of an immunomagnetic technique. Lymphocyte-depleted PBMC are then cultured for 7 days in RPMI medium, supplemented with 1% autologous human plasma and GM-CSF/IL-4, to generate dendritic cells. Dendritic cells are nonadherence as opposed to their monocyte progenitor. Thus, on day 7, non-adherent cells are harvested for further processing.

The dendritic cells derived from PBMC in the presence of GM-CSF and IL-4 are immature, in that they can lost the nonadherence property and revert back to macrophage cell fate if the cytokine stimuli are removed from the culture. The dendritic cells in an immature state are very effective in processing native protein antigens for the MHC class II restricted pathway (Romani et al. (1989) *J. Exp. Med.* 169:1169.

Further maturation of cultured dendritic cells is accomplished by culturing for 3 days in a macrophage-conditioned medium (CM), which contains the necessary maturation factors. Mature dendritic cells are less able to capture new proteins for presentation but are much better at stimulating resting T cells (both CD4+ and CD8+) to grow and differentiate.

Mature dendritic cells can be identified by their change in morphology, such as the formation of more motile cytoplasmic processes; by their nonadherence; by the presence of at least one of the following markers: CD83, CD68, HLA-DR or CD86; or by the loss of Fc receptors such as CD115 (reviewed in Steinman (1991) *Annu. Rev. Immunol.* 9:271.)

More specifically, the method requires collecting an enriched collection of white cells and platelets from leukapheresis that is then further fractionated by

countercurrent centrifugal elutriation (CCE) (Abrahamsen, T.G. et al. (1991) J. Clin. Apheresis. 6:48-53). Cell samples are placed in a special elutriation rotor. The rotor is then spun at a constant speed of, for example, 3000 rpm. Once the rotor has reached the desired speed, pressurized air is used to control the flow rate of cells. Cells in the elutriator are subjected to simultaneous centrifugation and a washout stream of buffer that is constantly increasing in flow rate. This results in fractional cell separations based largely but not exclusively on differences in cell size.

Quality control of APC and more specifically DC collection and confirmation of their successful activation in culture is dependent upon a simultaneous multi-color FACS analysis technique which monitors both monocytes and the dendritic cell subpopulation as well as possible contaminant T lymphocytes. It is based upon the fact that DCs do not express the following markers: CD3 (T cell); CD14 (monocyte); CD16, 56, 57 (NK/LAK cells); CD19, 20 (B cells). At the same time, DCs do express large quantities of HLA-DR, significant HLA-DQ and B7.2 (but little or no B7.1) at the time they are circulating in the blood (in addition they express Leu M7 and M9, myeloid markers which are also expressed by monocytes and neutrophils).

When combined with a third color reagent for analysis of dead cells, propridium iodide (PI), it is possible to make positive identification of all cell subpopulations (see Table 1):

TABLE 1
FACS analysis of fresh peripheral cell subpopulations

	Color #1 Cocktail 3/14/16/19/20/56/57	Color #2 HLA-DR	Color #3 PI
Live Dendritic cells	Negative	Positive	Negative
Live Monocytes	Positive	Positive	Negative
Live Neutrophils	Negative	Negative	Negative
Dead Cells	Variable	Variable	Positive

Additional markers can be substituted for additional analysis:

Color #1: CD3 alone, CD14 alone, etc.; Leu M7 or Leu M9; anti-Class I, etc.

Color #2: HLA-Dq, B7.1, B7.2, CD25 (IL2r), ICAM, LFA-3, etc.

The goal of FACS analysis at the time of collection is to confirm that the DCs are enriched in the expected fractions, to monitor neutrophil contamination, and to make sure that appropriate markers are expressed. This rapid bulk collection of enriched DCs from human peripheral blood, suitable for clinical applications, is absolutely dependent on the analytic FACS technique described above for quality control. If need be, mature DCs can be immediately separated from monocytes at this point by fluorescent sorting for "cocktail negative" cells. It may not be necessary to routinely separate DCs from monocytes because, as will be detailed below, the monocytes themselves are still capable of differentiating into DCs or functional DC-like cells in culture.

Once collected, the DC rich/monocyte APC fractions (usually 150 through 190) can be pooled and cryopreserved for future use, or immediately placed in short term culture.

Alternatively, others have reported that a method for upregulating (activating) dendritic cells and converting monocytes to an activated dendritic cell phenotype. This method involves the addition of calcium ionophore to the culture media convert monocytes into activated dendritic cells. Adding the calcium ionophore A23187, for example, at the beginning of a 24-48 hour culture period resulted in uniform activation and dendritic cell phenotypic conversion of the pooled "monocyte plus DC" fractions: characteristically, the activated population becomes uniformly CD14 (Leu M3) negative, and upregulates HLA-DR, HLA-DQ, ICAM-1, B7.1, and B7.2. Furthermore this activated bulk population functions as well on a small numbers basis as a further purified.

Specific combination(s) of cytokines have been used successfully to amplify (or partially substitute) for the activation/conversion achieved with calcium ionophore: these cytokines include but are not limited to G-CSF, GM-CSF, IL-2, and IL-4. Each cytokine when given alone is inadequate for optimal upregulation.

In one embodiment, the APCs and cells expressing one or more antigens are autologous. In another embodiment, the APCs and cells expressing the antigen are allogeneic, *i.e.*, derived from a different subject.

Presentation of Antigen by the APC

Peptide fragments from antigens must first be bound to peptide binding receptors (major histocompatibility complex class I and class II molecules) that display the antigenic peptides on the surface of the APCs. Palmer E. and Cresswell (1998) Annu. Rev. Immunol. **16**:323 and Germain R.N. (1996) Immunol. Rev. **151**:5. T lymphocytes produce an antigen receptor that they use to monitor the surface of APCs for the presence of foreign peptides. The antigen receptors on $CD4^+$ T cells recognize antigenic peptides bound to MHC class II molecules whereas the receptors on $CD8^+$ T cells react with antigens displayed on class I molecules. For a general review of the methods for presentation of exogeneous antigen by APC, see Raychaudhuri and Rock (1998) Nature Biotechnology **16**:1025.

For purposes of immunization, antigens can be delivered to antigen-presenting cells as protein/peptide or in the form of polynucleotides encoding the protein/peptide *ex vivo* or *in vivo*. The methods described below focus primarily on DCs which are the most potent, preferred APCs.

Several different techniques have been described to produce genetically modified APCs. These include: (1) the introduction into the APCs of polynucleotides that express antigen or fragments thereof; (2) infection of APCs with recombinant vectors to induce endogenous expression of antigen; and (3) introduction of tumor antigen into the DC cytosol using liposomes. (See, Boczkowski D. et al. (1996) J. Exp. Med. **184**:465; Rouse et al. (1994) J. Virol. **68**:5685; and Nair et al. (1992) J. Exp. Med. **175**:609). For the purpose of this invention, any method which allows for the introduction and expression of the heterologous or non-self antigen and presentation by the MHC on the surface of the APC is within the scope of this invention.

Several techniques have been described for the presentation of exogenous protein and/or peptide by the APC. These techniques are briefly described below.

Antigen Pulsing

Pulsing is accomplished *in vitro/ex vivo* by exposing APCs to antigenic protein or peptide(s). The protein or peptide(s) are added to APCs at a concentration of 1-10 μm for approximately 3 hours. Paglia et al. (1996) J. Exp. Med. **183**:317, has shown that APC incubated with whole protein *in vitro* were recognized by MHC class I-restricted CTLs, and that immunization of animals with these APCs led to the development of antigen-specific CTLs *in vivo*.

Protein/peptide antigen can also be delivered to APC *in vivo* and presented by the APC. Antigen is preferably delivered with adjuvant via the intravenous, subcutaneous, intranasal, intramuscular or intraperitoneal route of delivery. Grant E.P. and Rock K.L. (1992) J. Immunol. **148**:13; Norbury, C. C. et al. (1995) Immunity **3**:783; and Reise-Sousa C. and Germain R.N. (1995) J. Exp. Med. **182**:841.

Antigen Painting

Another method which can be used is termed "painting". It has been demonstrated that glycosyl-phosphotidylinositol (GPI)-modified proteins possess the ability to reincorporate themselves back into cell membranes after purification. Hirose et al. (1995) Methods Enzymol. **250**:582; Medof et al. (1984) J. Exp. Med. **160**:1558; Medof (1996) FASEB J. **10**:574; and Huang et al. (1994) Immunity **1**:607, have exploited this property in order to create APCs of specific composition for the presentation of antigen to CTLs. Expression vectors for β2-microglobulin and the HLA-A2.1 allele were first devised. The proteins were expressed in Schneider S2 *Drosophila melanogaster* cells, known to support GPI-modification. After purification, the proteins could be incubated together with a purified antigenic peptide which resulted in a trimolecular complex capable of efficiently inserting itself into the membranes of autologous cells. In essence, these protein mixtures were used to "paint" the APC surface, conferring the ability to stimulate a CTL clone that was specific for the antigenic peptide. Cell coating was shown to occur rapidly and to be protein concentration dependent. This method of generating APCs bypasses the need for gene transfer into the APC and permits control of antigenic peptide densities at the cell surfaces.

Foster Antigen Presenting Cells

Foster APCs are derived from the human cell line 174xCEM.T2, referred to as T2, which contains a mutation in its antigen processing pathway that restricts the association of endogenous peptides with cell surface MHC class I molecules (Zweerink et al. (1993) J. Immunol. **150**:1763). This is due to a large homozygous deletion in the MHC class II region encompassing the genes TAP1, TAP2, LMP1, and LMP2, which are required for antigen presentation to MHC class I-restricted $CD8^+$ CTLs. In effect, only "empty" MHC class I molecules are presented on the surface of these cells. Exogenous peptide added to the culture medium binds to these MHC molecules provided that the peptide contains the allele-specific binding motif. These T2 cells are referred to herein as "foster"

APCs. They can be used in conjunction with this invention to present the heterologous, altered or control antigen.

Transduction of T2 cells with specific recombinant MHC alleles allows for redirection of the MHC restriction profile. Libraries tailored to the recombinant allele will be preferentially presented by them because the anchor residues will prevent efficient binding to the endogenous allele.

High level expression of MHC molecules makes the APC more visible to the CTLs. Expressing the MHC allele of interest in T2 cells using a powerful transcriptional promoter (e.g., the CMV promoter) results in a more reactive APC (most likely due to a higher concentration of reactive MHC-peptide complexes on the cell surface).

Expansion of Immune Effector Cells

In one embodiment, the present invention makes use of these APCs to stimulate production of an enriched population of antigen-specific immune effector cells. The antigen-specific immune effector cells are expanded at the expense of the APCs, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) Molec. Med. Today 3: 261. The substantially pure populationof educated, antigen-specific immune effector cells produced by this method are useful to cause tumor regression.

The APCs (*e.g.*, DCs) presenting the heterologous/altered antigen are mixed with naïve immune effector cells. Preferably, the cells may be cultured in the presence of a cytokine, for example IL2. Because dendritic cells secrete potent immunostimulatory cytokines, such as IL-12, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In any event, the culture conditions are such that the antigen-specific immune effector cells expand (*i.e.*, proliferate) at a much higher rate than the APCs. Multiple infusions of APCs and optional cytokines can be performed to further expand the population of antigen-specific cells.